REMARKS

Claims 1-17 are pending in the present application. Claims 12-14 and 16 are withdrawn from consideration as being drawn to a non-elected invention. By virtue of this response, claim 5 has been cancelled. Claims 1, 6, and 10 have been amended. Upon entry of this amendment, claims 1-11, 15, and 17 are currently under consideration.

Support for the amendment of claim 1 can be found, for example, on previous claim 5 and page 36, lines 16-22 of the specification. Claims 6 and 10 are amended to correct informalities. No new matter is added.

With respect to all amendment to claims, Applicants have not dedicated or abandoned any unclaimed subject matter and, moreover, have not acquiesced to any rejections and/or objections made by the Office. Applicants expressly reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation, continuation-in-part, and/or divisional applications.

Claim Rejections – 35 USC § 102(b)

Claims 1-6, 10, 11, 15, and 17 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Capua et al. ("Capua," Veterinary Record 2000, Vol. 147, No 26 page 751). Applicants respectfully traverse this rejection.

As a preliminary matter, Applicants respectfully submit that, solely in an effort to expedite prosecution, claim 1 has been amended to recite "wherein the specimen comprises biological fluid from an animal which has been subjected to vaccination by means of a heterologous vaccine characterized by the same subtype of viral haemagglutinin HAx and a different subtype of neuraminidase NAz." Claims 2-4, 6, 10, 11, 15, and 17 depend from claim 1.

As the Examiner has acknowledged, Capua "does not teach specific assays to detect antibodies." Page 4, line 5 of the Office Action. Specifically, Capua is a brief proposal for methods of controlling avian influenza. It does not disclose contacting an antigen comprising or encoding an

amino acid sequence of a neuraminidase protein or a fragment thereof with a specimen of the biological fluid. Neither does Capua disclose determining whether an antigen has any antineuraminidase antibodies bound thereto. Capua also does not disclose a diagnostic method using a specimen comprising biological fluid from an animal which has been subjected to vaccination by means of a heterologous vaccine characterized by the same subtype of viral haemagglutinin HAx and a different subtype of neuraminidase NAz. By contract, the reference states,

> The presence of a different neuraminidase (N) subtype, which will induce specific antibodies (against N3 rather than N1), will enable us, with the aid of an *ad hoc* diagnostic kit, to discriminate between infected and vaccinated flocks.
>
> Page 751, middle column.

Thus, Capua focuses on discriminating between infected and vaccinated flocks rather than diagnosing avian influenza virus infection using a specimen comprising biological fluid from an animal which has been subjected to vaccination by means of a heterologous vaccine characterized by the same subtype of viral haemagglutinin HAx and a different subtype of neuraminidase NAz.

Moreover, at the time the present application was filed, the conventional method for detecting neuraminidase antibodies is a neuaminidase inhibition (NI) test. Rather than testing for the binding of an antigen to the antibody, the N1 test is a functional test based on the inhibition of neuraminidase enzyme activities. *See, e.g.*, page 470, last paragraph of the left-hand column of Swayne et al., *Rev. Sci. Tech. Off. Int. Epiz* 2000, 19(2):463-483 (Exhibit A). Accordingly, the mere mention of "an *ad hoc* diagnostic kit" does not inherently disclose the specific steps recited in the claim 1.

For at least the above reasons, Applicants respectfully submit that the claimed methods are novel over Capua et al. and request that the 35 U.S.C. § 102(b) rejection be withdrawn.

Claim Rejections – 35 USC § 103(a)

Claims 1 and 7-9 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Capua et al. and Van de Perre et al. ("Van de Perre," *J Clinical Micro.* 1988 Vol 26, pages 552-556). Applicants respectfully traverse.

Capua et al. is discussed above. Van de Perre is cited as allegedly disclosing specific assays for detecting antibodies. As discussed in further detail below, Capua and Van de Perre, alone or in combination, do not render the claimed methods obvious.

The Examination Guidelines for Determining Obviousness under 35 U.S.C. § 103 added in MPEP in view of the recent Supreme Court decision *KSR International Co. v. Teleflex*, 127 S. Ct. 1727 (2007), identifies seven rationales that can be used to support the legal conclusion of obviousness. MPEP 2141. One rationale identified in the Examination Guidelines is as follows:

> G. Some Teaching, Suggestion or Motivation in the Prior Art That Would Have Led One of Ordinary Skill To Modify the Prior Art Reference or To Combine Prior Art Reference or To Combine Prior Art Reference Teachings To Arrive at the Claimed Invention.
>
> To reject a claim based on this rationale, Office personnel must resolve the *Graham* factual inquiries. Office personnel must then articulate the following: (1) a finding that there was some teaching, suggestion, or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) a finding that there was reasonable expectation of success; and (3) whatever additional findings based on the *Graham* factual inquiries may be necessary, in view of the facts of the case under consideration, to explain a conclusion of obviousness.
>
> MPEP 2143.

Applicants respectfully submit that claims in the present invention are not obvious in view of the cited references. As discussed above, Claim 1 as amended recites "a diagnostic method for detecting infection with an avian influenza virus of a specific epidemic strain (HxNy) comprising the steps of: contacting an antigen with a specimen of biological fluid from an animal to be tested, wherein the antigen is a recombinant antigen comprising comprises or encodes an amino acid

sequence of a neuraminidase protein (NAy) or a fragment thereof; and determining whether the antigen has any anti-neuraminidase antibodies bound thereto by means of a positivity detection test, wherein the specimen comprises biological fluid from an animal which has been subjected to vaccination by means of a heterologous vaccine characterized by the same subtype of viral haemagglutinin HAx and a different subtype of neuraminidase NAz." The invention is based on the surprising finding that, vaccination with a heterologous vaccine still permits limited replication of the infecting field virus strain in the vaccinated bird. This minimal degree of replication is sufficient to generate detectable levels of antibody against the neuraminidase subtype of the field strain without compromising the health of the animal.

As discussed in the specification, one major problem with bird vaccination was the discrimination between birds which have merely been vaccinated and those which have been vaccinated but subsequently exposed to the live filed virus (i.e., the epidemic strain). The identification, and subsequent extermination, of the latter vaccinated, exposed birds is essential in the effective control of the spread of avian influenza, since they can act as "healthy carriers of the live field virus, thus leading to further outbreaks of infection. See page 8, line 10 to page 9, line 2 of the specification.

However, due to the characteristics of the viral replication cycle and the occurrence of cross-reactions among different types of neuraminidases, a person of ordinary skill in the art would not have understood that an antigen binding test for neuraminidase would confer sufficient sensitivity and specificity at the time of filing the present application. In addition, it was unpredicatable whether vaccine-induced neuraminidase antibodies would interfere with the production of anti-neuraminidase antibodies following subsequent infection with the field virus strain. The cited references are completely silent about diagnosing viral infection in vaccinated animals, let alone direct a person of ordinary skill in the art to develop a method claimed in the present application.

Accordingly, Applicants respectfully submit that Capua and Van de Perre, alone or in combination, do not render the claimed methods obvious. Applicants respectfully request that the 35 U.S.C. § 103 rejection be withdrawn.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 404172000300. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: November 13, 2008

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EXHIBIT A

Rev. sci. tech. Off. int. Epiz., 2000, 19 (2), 463-482

Highly pathogenic avian influenza

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Summary

Highly pathogenic (HP) avian influenza (AI) (HPAI) is an extremely contagious, multi-organ systemic disease of poultry leading to high mortality, and caused by some H5 and H7 subtypes of type A influenza virus, family *Orthomyxoviridae*. However, most AI virus strains are mildly pathogenic (MP) and produce either subclinical infections or respiratory and/or reproductive diseases in a variety of domestic and wild bird species. Highly pathogenic avian influenza is a List A disease of the Office International des Epizooties, while MPAI is neither a List A nor List B disease. Eighteen outbreaks of HPAI have been documented since the identification of AI virus as the cause of fowl plague in 1955.

Mildly pathogenic avian influenza viruses are maintained in wild aquatic bird reservoirs, occasionally crossing over to domestic poultry and causing outbreaks of mild disease. Highly pathogenic avian influenza viruses do not have a recognised wild bird reservoir, but can occasionally be isolated from wild birds during outbreaks in domestic poultry. Highly pathogenic avian influenza viruses have been documented to arise from MPAI viruses through mutations in the haemagglutinin surface protein.

Prevention of exposure to the virus and eradication are the accepted methods for dealing with HPAI. Control programmes, which imply allowing a low incidence of infection, are not an acceptable method for managing HPAI, but have been used during some outbreaks of MPAI. The components of a strategy to deal with MPAI or HPAI include surveillance and diagnosis, biosecurity, education, quarantine and depopulation. Vaccination has been used in some control and eradication programmes for AI.

Keywords

Avian diseases – Fowl pest – Fowl plague – Highly pathogenic avian influenza – Orthomyxoviruses.

Introduction

Highly pathogenic (HP) avian influenza (AI) is an extremely infectious, systemic viral disease of poultry that produces high mortality and necrobiotic, haemorrhagic or inflammatory lesions in multiple visceral organs, the brain and skin (4, 103). Highly pathogenic avian influenza and fowl plague are synonymous terms, the latter being the historical designation for the disease originally described in domestic fowl (*Gallus gallus domesticus*), and the former the more accurate currently accepted name for the disease in all bird species (12, 73). Fowl plague has been known by other names including fowl pest (*peste aviaire*), *Geflügelpest*, Brunswick bird plague, Brunswick disease, fowl disease, and fowl or bird grippe (98).

History

Fowl plague was first reported in Italy in 1878 by Perroncito who described a severe, rapidly spreading disease that produced high mortality in chickens (98). In 1880, Rivolto and Delprato differentiated fowl plague from the clinically similar septicaemic form of fowl cholera and used the name *typhus exudatious galliniarum* to describe fowl plague (98). The disease spread throughout Europe in the late 1800s and early 1900s via poultry exhibitions and shows, where it became endemic in domestic poultry until the 1930s. In 1901, the cause was determined to be a filterable agent, a virus, although only in 1955 was the virus identified and classified as Type A influenza virus (orthomyxovirus), and shown to be related to other influenza viruses that commonly

infected humans, pigs and horses (82). From the 1960s onwards, a variety of influenza viruses were isolated from turkeys with milder disease (i.e. non-fowl plague syndromes), and these viruses were termed mildly pathogenic (MP) AI viruses or AI viruses of low pathogenicity (108). The agar gel immunodiffusion (AGID) test became the international standard for serological diagnosis and surveillance (16). In 1972, wild waterfowl of the order *Anseriformes* (ducks and geese) were demonstrated to be a principal reservoir and the natural host for MPAI viruses (90). In 1979, the cleavability of the haemagglutinin (HA) protein was identified as the major determinant of virulence in HPAI viruses (21). In 1981, the first International Symposium on avian influenza was convened in Beltsville, Maryland, United States of America (USA), and the term 'fowl plague' was abandoned for the more accurate term 'highly pathogenic avian influenza' (12).

Avian influenza virus

Description of the aetiological agent

Avian influenza viruses are negative-sense, segmented, ribonucleic acid viruses of the family *Orthomyxoviridae*. The *Orthomyxoviridae* family includes several segmented viruses including the Type A, B and C influenza viruses. The Type A influenza viruses, which include all AI viruses, can infect a wide variety of animals including wild ducks, chickens, turkeys, pigs, horses, mink, seals and humans. The type B and C viruses primarily infect only humans and occasionally pigs. The different types of influenza viruses can be differentiated serologically based on antigenic differences in the conserved internal proteins, primarily the nucleoprotein and matrix genes, using the AGID test (16).

Type A influenza viruses have eight gene segments that encode ten different proteins (56). The proteins can be divided into surface proteins and internal proteins. The surface proteins include the HA, neuraminidase (NA) and matrix 2 proteins. The HA and NA proteins provide the most important antigenic sites for the production of a protective immune response, primarily in the form of neutralising antibody. However, these proteins have large antigenic variation, with fifteen HA and nine NA subtypes being recognised, based on haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests, respectively. The internal proteins include the polymerase complex, including the three polymerase proteins (PB1, PB2 and PA), the nucleoprotein, the matrix 1 protein, and non-structural proteins 1 and 2.

Definition of pathogenicity

The pathogenicity of individual AI viruses varies and should be determined in order to develop prevention, control and eradication strategies. The usage of the term HP implies that the virus is highly virulent for chickens and has been demonstrated to meet one or more of the following three criteria (72, 115):

a) any influenza virus that is lethal for six, seven or eight of eight (≥75%) four- to six-week-old susceptible chickens within ten days following intravenous inoculation with 0.2 ml of a 1:10 dilution of a bacteria-free, infectious allantoic fluid

b) any H5 or H7 virus that does not meet the criteria in *a)*, but has an amino acid sequence at the HA cleavage site that is compatible with HPAI viruses

c) any influenza virus that is not an H5 or H7 subtype and that kills one to five of eight inoculated chickens and grows in cell culture in the absence of trypsin.

Fulfilment of one or more of the above criteria would categorise the virus as an HPAI virus. In contrast, AI viruses that lack these three criteria are categorised as non-HPAI, and include AI viruses historically designated as MP, non-pathogenic, not pathogenic or of low pathogenicity, based on <75% lethality in experimental studies with chickens. 'Non-pathogenic' or 'not pathogenic' are usually used to denote strains that cause no clinical signs or deaths in experimentally inoculated chickens, while designations of MP or of low pathogenicity have been used to signify AI virus strains that typically caused some clinical signs, but mortality of less than 75% in experimental studies with chickens (one to five of eight inoculated chickens). Non-HPAI viral strains comprise all AI viruses of the H1–4, H6, and H8–15 subtypes, and most of those of H5 and H7 subtypes. Only a small number of AI viruses of the H5 and H7 HA subtypes have been HPAI viruses. Historically, 'fowl plague' was associated only with the H7 HA subtype. The outbreak of 1959 among chickens in Scotland was the first documentation of an HPAI virus of the H5 subtype (Table I).

The definition of AI adopted by the European Union (EU) is as follows: 'Avian influenza' means an infection of poultry caused by an influenza A virus which has an intravenous pathogenicity index in six-week-old chickens greater than 1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the HA (5).

The phrase 'highly pathogenic for chickens' does not indicate or imply that the AI virus strain is HP for other birds species, especially wild ducks or geese (*Anseriformes*). However, if a virus is HP for chickens, the virus will usually be HP for other birds within the order *Galliformes*, family *Phasianidae*, such as turkeys (*Meleagris gallopavo*) and Japanese quail (*Coturnix japonica*) (8). To date, HPAI viruses for chickens are generally non-pathogenic for ducks and geese in experimental studies (8, 35). Non-HPAI or MPAI viruses have been isolated from domestic ducks and geese in association with disease (usually respiratory disease), and mild-to-moderate mortality. However, these AI viruses do not produce high mortality in

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Table I
Documented outbreaks of highly pathogenic avian influenza since discovery of avian influenza as the cause of fowl plague in 1955 (4, 76)

Avian influenza virus	Subtype	Type and number of birds affected with high mortality or depopulated [a]	References
A/chicken/Scotland/59	H5N1	2 flocks of chickens (*Gallus gallus domesticus*). Total number of birds affected not reported	77; D.J. Alexander, personal communication
A/tern/South Africa/61	H5N3	1,300 common terns (*Sterna hirundo*)	19
A/turkey/England/63	H7N3	29,000 breeder turkeys (*Meleagridis gallopavo*)	123
A/turkey/Ontario/7732/66	H5N9	8,100 breeder turkeys	58
A/chicken/Victoria/76	H7N7	25,000 laying chickens, 17,000 broilers and 16,000 ducks (*Anas platyrhynchos*)	11, 113
A/turkey/England/199/79	H7N7	3 commercial farms of turkeys. Total number of birds affected not reported	3, 6
A/chicken/Pennsylvania/1370/83	H5N2	17 million birds in 452 flocks; most were chickens or turkeys. a few chukar partridges (*Alectoris chukar*) and guinea-fowl (*Numida meleagris*)	34, 114
A/turkey/Ireland/1378/83	H5N8	800 meat turkeys died on original farm; 8,640 turkeys, 28,020 chickens and 270,000 ducks were depopulated on original and 2 adjacent farms	4, 61
A/chicken/Victoria/85	H7N7	24,000 broiler breeders, 27,000 laying chickens, 69,000 broilers and 118,518 chickens of unspecified type	14, 28
A/turkey/England/50-92/91	H5N1	8,000 turkeys	8
A/chicken/Victoria/92	H7N3	12,700 broiler breeders and 6,700 ducks	84, 124
A/chicken/Queensland/95	H7N3	22,000 laying chickens	124
A/chicken/Puebla/8623-607/94 A/chicken/Queretaro/14588-19/95	H5N2	Chickens [b]	118
A/chicken/Pakistan/447/95 A/chicken/Pakistan/1369-CR2/95	H7N3	3.2 million broilers and broiler breeder chickens [c]	69
A/chicken/Hong Kong/220/97	H5N1	1.4 million chickens and various lesser numbers of other domestic birds in contact with the chickens on farms and in the live-bird market system	88, 99
A/chicken/New South Wales/1651/97	H7N4	128,000 broiler breeders, 33,000 broilers and 251 emu (*Dromaius novaehollandiae*)	76
A/chicken/Italy/330/97	H5N2	2,116 chickens, 1,501 turkeys, 731 guinea-fowl, 2,322 ducks, 204 quail (species unknown), 45 pigeons (*Columbia livia*), 45 geese (species unknown) and 1 pheasant (species unknown)	24
A/turkey/Italy/99	H7N1	297 farms; 5.8 million laying chickens, 2.2 million meat and breeder turkeys, 1.6 million broiler breeders and broilers, 156,000 guinea-fowl, 168,000 quail, 577 backyard poultry and 200 ostriches [d]	I. Capua, personal communication

a) Most outbreaks were controlled by 'stamping out' or depopulation policies for infected and/or exposed populations of birds. Chickens, turkeys and birds of the order *Galliformes* had clinical signs and mortality patterns consistant with highly pathogenic avian influenza, while ducks, geese and other birds lacked or had low mortality rates or infrequent presence of clinical signs.
b) A 'stamping-out' policy was not used for control. The outbreak of AI had concurrent circulation of mildly pathogenic avian influenza and highly pathogenic avian influenza (HPAI) virus strains. However, HPAI virus strains were present only from late 1994 to mid-1995. Estimates of the number of birds infected with HPAI strains are unavailable, but 360 commercial chicken flocks were 'depopulated' for AI in 1995 through controlled marketing.
c) A 'stamping-out' policy was not used for control. Surveillance, quarantine, vaccination and controlled marketing were used as the control strategy. The numbers affected are crude estimates.
d) The outbreak began on 17 December 1999 and was on-going at the time of writing on 15 February 2000. A 'stamping-out' policy was in force as the control method. Estimates given include birds present in outbreaks, either affected or depopulated.

experimental studies with chickens, ducks or geese (4). In one report, an H5N1 AI virus was isolated from a sick domestic goose in the People's Republic of China and the virus was HP for chickens based on the sequence of the viral HA proteolytic cleavage site, as listed in criterion *b)* above (128).

Overview of highly pathogenic avian influenza

Outbreaks since 1955

Eighteen outbreaks of HPAI have been documented in the English-language literature since the identification of influenza viruses as the cause of HPAI in 1955 (4, 76). This includes seventeen outbreaks in domestic poultry and one in wild common terns (*Sterna hirundo*) (Table I). In addition, two AI virus strains recovered from non-domestic birds have been reported to be HP for chickens, namely: A/finch/Germany/72 (H7N1) and A/gull/Germany/79 (H7N7) (4). The origins of the former virus remain unclear, but the latter was possibly the result of spread from poultry, as outbreaks occurred in Eastern Europe at that time (D.J. Alexander, personal communication, 15 December 1999). The recent outbreaks of HPAI have occurred in North America, Europe, Asia and Australia (Table I). Prior to 1955, HPAI outbreaks had been reported in Africa and South America (33, 98). No outbreaks have been reported in Antarctica. Although serological evidence of infection by AI viruses has been reported for penguins, such infections were most probably not from HPAI viruses (67).

Highly pathogenic avian influenza is not endemic in commercial domestic poultry, but has produced local/regional epizootics in chickens and turkeys on large commercial farms, in meat and breeder turkeys on smaller

commercial farms, and in poultry on farms and in retail facilities that provide poultry for the live-bird market system (Table I). Of the seventeen HPAI outbreaks which have occurred in poultry since 1955, seven have affected over 100,000 birds each, while the remaining ten affected less than 100,000 birds each (Table I). In sixteen outbreaks, the HPAI viruses were eliminated and in the current H7N1 outbreak in Italy, eradication is underway. The total number of domestic poultry affected by HPAI has been a small percentage of the total world poultry production of 22 billion birds per year, or approximately 750 billion birds since 1955.

Impact on trade

Assessment of pathogenicity of a newly isolated AI virus is critical for development of appropriate control strategies and to assess the impact on international trade. Highly pathogenic avian influenza is a List A disease of Office International des Epizooties (OIE) and has been used as a legitimate trade barrier to protect countries or regions from introduction of an exotic or foreign poultry disease, i.e. HPAI. However, reports of isolation of MPAI viruses from domestic and non-domestic birds, or serological evidence of AI virus infection in non-poultry species have been used by some countries as non-tariff trade barriers for limiting importation of poultry and poultry products. Mildly pathogenic avian influenza is neither a List A nor a List B disease of the OIE.

Ecology

Influenza viruses in birds and mammals

Influenza viruses can infect a wide variety of birds and mammals, thus demonstrating an unusually wide host range. While influenza appears to be well adapted to the natural host (wild aquatic birds), causing little, if any disease, when influenza viruses infect non-host species, disease can often result. Serious disease outbreaks have been reported for poultry species, primarily chickens and turkeys, as well as mammalian species such as pigs, horses, mink, seals and humans. Although influenza viruses which become better adapted for a particular species are considered to be pathogens of that species only, numerous exceptions have occurred. For example, classic-H1N1 swine influenza viruses generally only infect pigs, but this lineage of virus has been known to infect humans and turkeys, often causing serious disease (10, 42, 126). The genetic determinants for species specificity of different influenza viruses are currently not known, although multiple genes appear to play some role in the process (92, 111).

Mildly pathogenic avian influenza viruses in wild birds

In contrast to the geographically restricted and limited number of outbreaks due to HPAI viruses, MP (non-HP) AI viruses are world-wide in distribution, predominantly infecting a variety of wild bird species, but also some domestic birds. Isolation of influenza from wild birds has been documented from five continents including North America, Europe, Asia, Africa and Australia (19, 52, 60, 67, 90, 94, 101, 112). Serological evidence has also been reported in wild birds from Antarctica (67).

The natural host species and reservoir for influenza viruses are wild aquatic birds, including ducks, gulls and other shorebirds (50, 90). Many of these birds undergo annual long distance migrations. This wild bird reservoir is also the original source of all viral genes for both avian and mammalian lineages of influenza viruses. For example, all fifteen HA and nine NA subtypes of influenza have been found in wild birds (121). Avian species from at least nine different orders of birds have been reported to be naturally and asymptomatically infected with influenza viruses, but the main reservoir is believed to be in birds belonging to the orders *Anseriformes* and *Charadriiformes* (93). The *Anseriformes* include ducks, geese and swans, and *Charadriiformes* include gulls, terns, surfbirds and sandpipers. Although influenza viruses occur commonly in both orders of birds, most epidemiological studies have been conducted on mallard ducks (86, 87, 90, 95, 112). Although MPAI viruses can be detected in ducks at most times of the year, the highest percentage of birds shedding virus is found in the pre-migration stage in late summer (93). Most HA and all the NA subtypes have been isolated from wild ducks, but certain HA and NA subtypes appear to occur much more frequently, including the HA subtypes H3, H6 and H4, and the NA subtypes N8, N4 and N6 (87). However, the percentage of each subtype that is isolated can vary widely depending on the time of year when the samples are taken, and the geographic location.

Although influenza viruses can infect large numbers of wild waterfowl, infection usually only produces an asymptomatic enteric infection (26, 51, 119). Infected birds can produce large amounts of virus, often defecated directly into the water. The contamination of the aquatic environment appears to be an efficient method of transmission of virus to susceptible wild birds or to domestic birds which share the same environment. Water contaminated with AI virus in the faeces of infected wild ducks has been a source of infection for domestic turkeys (89).

Mildly pathogenic avian influenza in domestic birds

Chickens and turkeys are not natural host species for AI viruses. In two different studies of wild turkeys, no serological evidence of infection was observed (29, 45). Although no comparable studies have been performed on red jungle fowl, the ancestor of modern chickens, this species is unlikely to play a role in the maintenance of influenza in the environment. Although chickens and turkeys are not natural hosts for AI viruses, these birds can readily become infected with the virus. The direct transmission of influenza viruses from migrating waterfowl to range-reared turkeys has been

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implicated as a common source of infection in the USA (41), and probably also plays a major role in transmission of AI to chickens. However, transmission of influenza from wild waterfowl to chickens and turkeys may also occur through intermediates, in particular domestic ducks and geese that are reared or marketed in close association with chickens and turkeys. Once influenza viruses have been introduced into commercial poultry operations, the virus can often spread rapidly to new locations or be maintained for long periods of time (23, 109).

In addition to chickens and turkeys, MPAI viruses have been isolated from domestic ducks, domestic and imported exotic birds, ratites and occasionally quail and game birds (4). Mildly pathogenic avian influenza virus infections in the latter two groups of birds have been detected most frequently in domestic poultry in live bird markets of large cities. Although AI infection in these birds may be asymptomatic, outbreaks of respiratory disease with mortality have been reported (7, 46, 128). Transmission of AI viruses in poultry is through respiratory secretions and faeces.

Highly pathogenic avian influenza viruses

No wild bird reservoir has been identified for HPAI viruses. Except for the single outbreak of HPAI in common terns in South Africa (Table 1), HPAI has been rarely isolated from wild birds even on farms experiencing outbreaks of HPAI in domestic poultry (70). Extensive surveys for AI viruses in wild habitats of waterfowl in North America have identified thousands of AI viruses, all MP viruses (4). In the outbreaks of 1983-1984 in Pennsylvania and 1994-1995 in Mexico, HPAI viruses arose following the mutation of H5 MPAI virus which circulated widely in domestic poultry (49, 75). An experimental chicken embryo model has shown that some H5 and H7 MPAI viruses can mutate to form HPAI viruses that are similar pathobiologically and molecularly to HPAI field virus (107). This data suggests that HPAI viruses arise in domestic poultry populations from MPAI viruses and are not widely maintained as HPAI in wild bird populations. However, this does not preclude that small groups of wild birds could serve as reservoirs of HPAI viruses derived from poultry.

The infection and disease

Most HPAI virus strains from domestic poultry have been isolated from turkeys and chickens, but infection, clinical signs and high mortality have been reported in diverse genera and species within the order *Galliformes*, family *Phasianidae*, including Japanese quail, helmeted guinea-fowl (*Numida meleagris*), chukar partridges (*Alectoris chukar*), northern bobwhite quail (*Colinus virginianus*) and common pheasants (*Phasianus colchicus*) (24, 33, 78). Highly pathogenic avian influenza is the result of systemic replication of the virus and cell death in a variety of visceral organs, brain and skin. Poultry flocks infected with HPAI virus have high morbidity and mortality rates with birds developing severe clinical signs, often with rapid death.

In contrast, the majority of AI virus strains are non-HP under natural or experimental conditions, and produce either subclinical infections or mild-to-moderate disease syndromes affecting the respiratory, urinary and reproductive systems (4, 103). These viruses replicate locally, predominantly in the respiratory and alimentary tracts. Generally, the mortality rates due to MPAI are low compared to HPAI, but mortality rates can be high when MPAI is accompanied by secondary viral or bacterial pathogens (71). In this paper, the emphasis will be placed on the epidemiological and pathobiological features of HPAI, while MPAI will be a minor component and used for comparative purposes with HPAI. Previous papers have provided more detailed comparisons of lesions of poultry infected with either MPAI or HPAI viruses (44, 65, 103).

Clinical signs

Chickens and turkeys with HPAI are typically found dead with few clinical signs other than depression, recumbency and a comatose state (34, 43, 48, 54, 65, 98). Close observation of remaining birds has revealed reduced activity, decreased sensitivity to stimuli, reduction in 'house noise', dehydration and decreased feed intake which rapidly progressed to severe depression and death. The older the birds, the greater the frequency of clinical signs appearing before death. In breeders and laying chickens, egg production drops precipitously to near zero after three to five days. Occasionally, torticollis, paresis, paralysis, excitation, convulsions and rolling or circling movements are noted in a few birds that survive to the subacute stage of the infection. Diarrhoea may be evident as bile- or urate-stained loose droppings with variable amounts of intermixed mucus. Respiratory signs such as nasal discharge, rales, coughing, sneezing or respiratory distress have been infrequently reported with HPAI (14), but are common with MPAI. Difficulty in breathing has been reported in birds affected by HPAI, with severe oedema of the upper respiratory tract or lungs (98).

Gross observations

The appearance of gross lesions is variable depending on the virus strain, the length of time from infection to death, and the age and species of poultry affected (33, 44, 103). In general, clinical signs, lesions and death have been seen with domestic poultry of the order *Galliformes*, family *Phasianidae*, but not for birds of the orders *Anseriformes* or *Charadriiformes* when infected with HPAI viruses.

In most cases of peracute infections with death (days one to two of infection), poultry have lacked visible gross lesions (44). However, some strains, such as the A/chicken/Hong Kong/220/97 (H5N1) and A/chicken/Italy/330/97 (H5N2), have caused severe lung lesions of congestion, haemorrhage and oedema in chickens, such that the excised tissue exudes

serous fluid and blood (Fig. 1a) (24, 99). Oedema of the brain has also been reported (99).

During the acute stages of infection with death (days three to five of infection), chickens have ruffled feathers, congestion and/or cyanosis of the comb and wattles, and swollen heads, especially prominent from periorbital and intramandibular subcutaneous oedema (Fig. 1b) (1, 44, 54, 103). Some viruses produced hyperaemia and oedema of the eyelids, conjunctiva and trachea (14). In birds which die, generalised congestion and haemorrhage may occur (44). Lesions are common in the combs and wattles, especially in adult chickens, and include petechial-to-ecchymotic haemorrhages, swelling from oedema and eventually depressed dark red-to-blue areas of ischaemic necrosis as the result of vascular infarction (Figs 1c and 1d). Subcutaneous haemorrhages and oedema may be present around the hock, on the shanks and feet (Fig. 1e) and occasionally on feathered skin all over the body. Some HPAI viruses, such as A/Queretaro/14588-19/95 (H5N2), commonly cause thickening of the skin over the distal legs with gelatinous oedema (Fig. 1f) (103). Petechial-to-ecchymotic haemorrhages may be present in multiple visceral organs or on the serosal surface, such as the epicardium of the heart (Fig. 1g), serosa of small intestine, abdominal fat, serosa of sternum, caecal tonsils, Meckel's diverticulum, Peyer's lymphoid patches of the small intestine (Fig. 1h), proventriculus around the glandular ducts or between glands (Fig. 1i), under the cuticle of the ventriculus and interfascicular regions of skeletal muscle. Primary lymphoid organs such as cloacal bursae and thymuses are severely atrophic, while the spleen may be normal in size or enlarged. Occasionally, spleens have white foci of necrosis. The pancreas may have red to light orange to brown mottling (44). Ruptured ova with 'yolk peritonitis' have been reported in layers and broilers and turkey breeders (1, 58).

Histopathology and immunohistochemical localisation of the virus

Histological lesions usually vary in severity and location, but typically include necrosis, haemorrhage and/or inflammation within multiple visceral organs; especially the heart (Fig. 1j), brain (Fig. 1k), adrenal and pancreas (Fig. 1l); and skin (1, 54, 64, 103, 117). Variation in the distribution and severity of lesions is the result of differences between strains of HPAI virus and species of bird.

In peracute deaths caused by HPAI viruses, necrosis and inflammation is generally lacking in parenchymal cells of most visceral organs, brain and skin. If present, such lesions are mild and multifocal in distribution. Most consistently, the HPAI virus replicates in the vascular endothelial cells (Fig. 1m) and cardiac myocytes (Fig. 1n), causing generalised cell death (99).

In acute infections with HPAI virus, the predominant lesions are necrosis, and to a lesser extent, apoptotic cell death with associated inflammation, haemorrhage and oedema (44). The longer the birds survive, the more prominent the inflammation and the less prominent the necrosis or apoptosis.

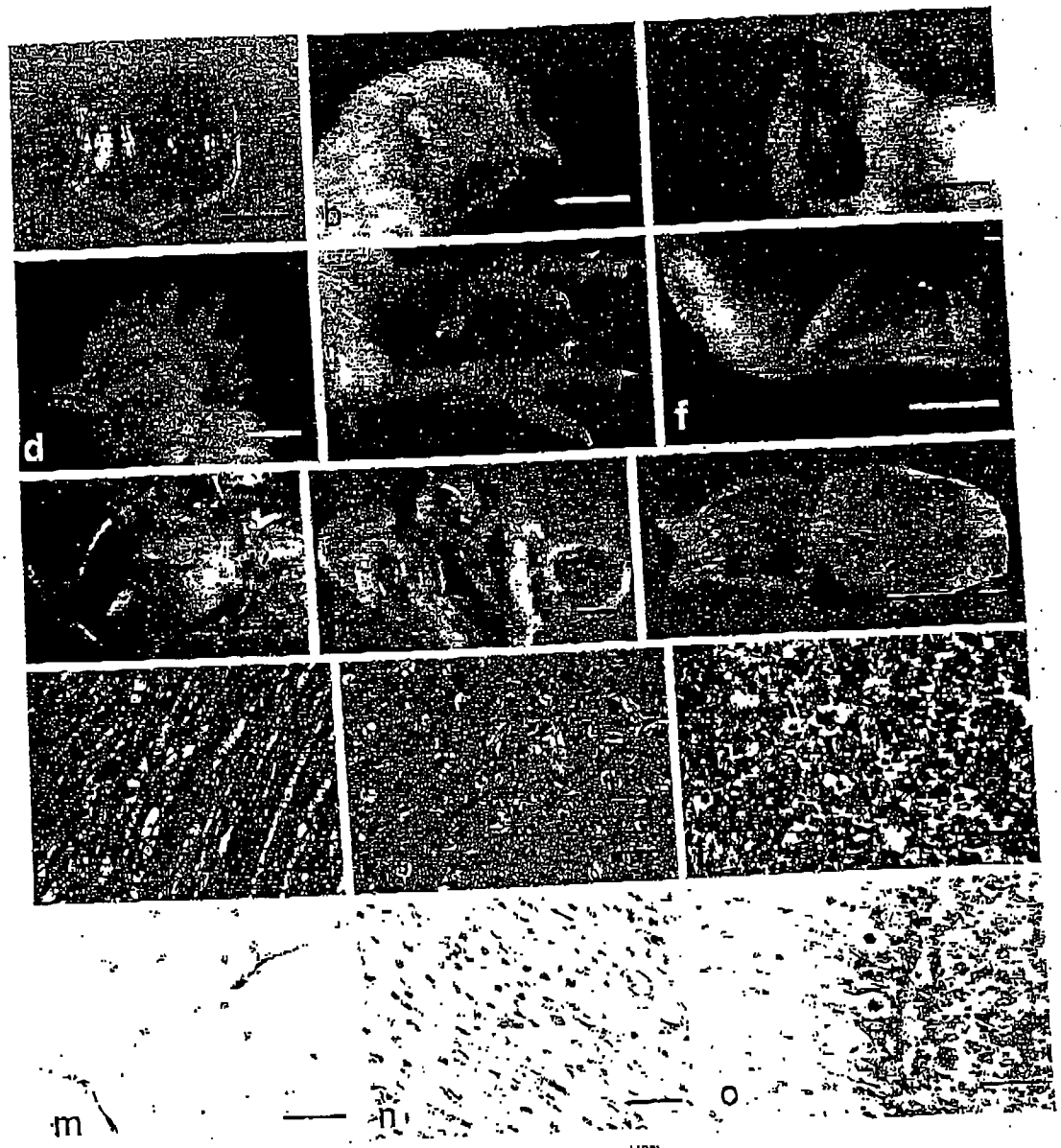
Lesions are most prominent in the brain, heart, pancreas, lung, adrenal and skin, but similar lesions have been reported in most visceral organs. Typical lesions include lymphohistiocytic meningoencephalitis with vasculitis and focal rarefication, widespread caseous necrosis of the pancreas, dermal vasculitis with thrombosis and infarction, lymphohistiocytic myocarditis with hyalin necrosis of myofibres and severe lymphocytic apoptosis of primary and secondary lymphoid tissues. The cells that are necrotic have associated AI viral replication (Fig. 1o), while the apoptotic lymphocytes do not have demonstrable AI viral antigen.

Epidemiology and diagnostic tests

A disease outbreak is usually the first indication of an infection with AI virus, and a combination of virus isolation, serological tests, and direct antigen detection is often used to detect infected flocks (108). Isolation of influenza viruses has been achieved by direct inoculation of nine- to eleven-day-old embryonating chicken eggs with homogenates from the lung, trachea, faeces and internal organs. Alternative methods of virus isolation have been used, primarily cell culture. Influenza viruses have been routinely isolated from clinical samples, however failures have occurred due to bacterial or viral contamination, incorrect storage of samples, inadequate samples or sample numbers, or because the infected birds were sampled after viral shedding had ceased. Once a virus has been isolated, the HA and NA subtypes are determined typically using the HI and NI tests (108). These tests require a panel of reagents specific for each HA and NA subtype. The virulence of the virus isolates can be tested using the previously described standardised intravenous pathogenicity test (115).

Following diagnosis of an outbreak of influenza, efforts to control the outbreak rely upon epidemiological tracking to determine the source of the outbreak and the extent to which the virus has been disseminated. Since influenza viruses can vary greatly in pathogenicity and more than one subtype of influenza may be circulating at the same time, the use of HA and NA subtyping of viruses responsible for an outbreak has become an important tool to track viruses. To thoroughly evaluate an outbreak, virus isolation and serology of infected birds must both be analysed.

The serological response of potentially infected birds remains the other important prerequisite tool in an eradication effort. Flocks are usually tested as a group, rather than testing all the individual birds. Often ten to thirty birds are randomly



a) Severe pulmonary oedema and haemorrhage in the lung. A/Hong Kong/156/97 (H5N1). (Bar = 1 cm) (99)
b) Severe swelling of the head, comb and wattle from subcutaneous oedema, A/chicken/Queretaro/14589-660/94 (H5N2). (Bar = 2 cm) (103)
c) Severe oedema, necrosis and haemorrhage of comb and wattles, highly pathogenic embryo derivative, A/chicken/NJ/12508/86 (H5N2). (Bar = 5 cm) (33)
d) Serous conjunctivitis with oedema of comb, wattles and periorbital area and necrosis of tips of comb. A/chicken/Puebla/8623-607/94 (H5N2). (Bar = 2 cm)
e) Severe subcutaneous haemorrhage and oedema of feet and leg shanks, A/Hong Kong/156/97 (H5N1). (Bar = 2 cm) (99)
f) Thickened dermis from oedema of distal leg. A/chicken/Queretaro/14589-660/94 (H5N2). (Bar = 2 cm)
g) Petechial haemorrhages in epicardial fat, A/chicken/NJ/12508/86 (H5N2). (Bar = 3 cm) (33)
h) Haemorrhage in lymphoid tissue of Peyer's patches and Meckel's diverticulum of the jejunum, A/Hong Kong/220/97 (H5N1). (Bar = 1 cm)
i) Submucosal haemorrhage surrounding ducts of glands in proventriculus, A/chicken/Hong Kong/156/97 (H5N1). (Bar = 2 cm) (99)
j) Non-suppurative myocarditis with necrosis of individual myocytes, A/chicken/Queretaro/14589-660/94 (H5N2). (Bar = 50 µm)
k) Focus of neuron necrosis in cerebrum, A/Hong Kong/156/97 (H5N1). (Bar = 50 µm)
l) Severe acute necrosis of pancreatic acinar glands. A/Hong Kong/156/97 (H5N1). (Bar = 50 µm)
m) Avian influenza viral antigen in the cytoplasm and nuclei of endothelial cells in the cerebellum. A/Hong Kong/156/97 (H5N1). (Bar = 30 µm) (99)
n) Avian influenza viral antigen in the cytoplasm and nuclei of cardiac myocytes, A/Hong Kong/156/97 (H5N1). (Bar = 25 µm)
o) Avian influenza viral antigen in the cytoplasm and nuclei of Purkinje neurons, Bergmann's glial cells and granular cells of the cerebellum, A/Hong Kong/156/97 (H5N1). (Bar = 50 µm)

Fig. 1
Gross (a-l), histological (j-l) and immunohistochemical (m-o) changes in chickens following experimental infection with highly pathogenic avian influenza viruses

selected from a suspect flock and the birds are tested with a type-specific influenza detection test. Two different tests are primarily used for the detection of type-specific antibodies, including the AGID and the enzyme-linked immunosorbent assay (ELISA).

The AGID test uses influenza antigens derived from the chorioallantoic membranes from infected embryonated chicken eggs, and antibodies to both the nucleoprotein and matrix 1 AI viral proteins are detected (16). The main advantages of the AGID test is that the test is relatively simple to perform and requires no sophisticated equipment. However, the AGID test is not as sensitive as the ELISA and typically requires an incubation step of two days before the results can be determined (63, 91).

Several different types of ELISA have been developed including two commercial indirect ELISAs for chickens, and competitive ELISAs for all bird species (18, 130). Both tests rely upon the detection of antibody against nucleoprotein. The indirect ELISA uses purified nucleoprotein as the antigen bound to the ELISA plate, the test serum is applied and any anti-nucleoprotein antibody present will bind to the antigen and will not be removed during the washing steps. A secondary antibody specific for the animal being tested is attached to an enzyme that allows for quantitative measure of the bound antibody (2, 63). The competitive ELISA is similar to the indirect assay in that the nucleoprotein antigen is bound to the ELISA plate, but the test serum competes for binding to the nucleoprotein with an anti-nucleoprotein monoclonal antibody. The enzyme-linked secondary antibody is specific for the monoclonal antibody, and the results of the test are inversely related to the strength of the colorimetric reaction (85, 130). The primary advantage of this test is that any antibody subtype can be detected and sera from almost any bird or mammal species can be tested.

If positive type A influenza serum samples are found, the samples are further tested to determine the HA and NA subtype specificity of the antibody response with the HI and NI tests using a panel of reagents to differentiate the fifteen HA- and nine NA subtypes (108). The determination of the HA and NA subtypes of the infecting virus provides additional confirmation of the outbreak virus and provides a convenient tracking tool. In some cases, only the HA subtyping test is necessary if an epizootic of a known subtype is occurring. Serological tests on a flock basis are considered a useful tool for determining whether a flock has been infected with influenza, but serological tests may miss a recent flock infection, because a measurable antibody titre does not develop until after approximately one week (108). An alternative method to determine if a flock of birds is still actively shedding virus is to introduce sentinel birds into the flock. Sentinel birds are specific-pathogen-free (SPF) birds susceptible to influenza or another pathogenic organism that allows for these naive birds to be monitored by virus isolation or seroconversion.

An alternative test which is used in conjunction with the serological tests is the antigen capture ELISA. This commercially available diagnostic test can detect Type A influenza viral nucleoprotein directly from clinical samples, usually from a cloacal or tracheal swab. The test provides a rapid and simple test to provide field identification of infected birds (30). However, the test will not differentiate AI viruses by HA or NA subtype and the cost per test is relatively high compared to other diagnostic tests.

Current evaluations of influenza disease outbreaks often rely upon sequencing of multiple genes of the virus. This sequence information provides several important pieces of information, some of which cannot be determined by any other method. The sequence data has two primary uses, firstly the determination of the relationships of the virus to previous or concurrent outbreaks can be determined. Although the HA and NA subtyping test can provide important clues about the parentage of a virus, outbreaks with no direct classic epidemiological links cannot be unambiguously determined. For example, three separate H5N2 outbreaks have occurred in North America in the 1980s and 1990s, and phylogenetic analysis of multiple genes demonstrates that these outbreaks were the result of separate introductions of virus (Fig. 2) (37). Phylogenetic analysis and the use of direct sequence comparisons between isolates to identify unique sequence markers provide the most detailed examination of influenza viruses. It is also important to evaluate multiple genes, since influenza viruses are segmented and reassort commonly. Two viruses with the same HA gene may have different internal genes (37, 100).

The second principal reason to sequence influenza isolates is to determine virulence traits from nucleotide sequences, which is the basis of criterion *b*, in the section 'Pathogenicity determination', for defining HPAI viruses. Currently, only the HA cleavage site can be evaluated in this way, but additional virulence genotypes should eventually become available. The sequence of the HA cleavage is most important for the evaluation of influenza viruses of the H5 and H7 subtypes, the only subtypes associated with the HP form of the virus. If multiple basic amino acids are present at the HA cleavage site, this indicates the potential for the virus to be HP or to become HP, and necessitates a more vigorous control programme.

Differentiation from closely related agents

The previously described clinical features, and gross and histological lesions are not diagnostic of HPAI (108). Similar features and lesions have been reported for highly virulent forms of Newcastle disease (velogenic Newcastle disease) and occasional acute cases of bacterial septicaemia, especially

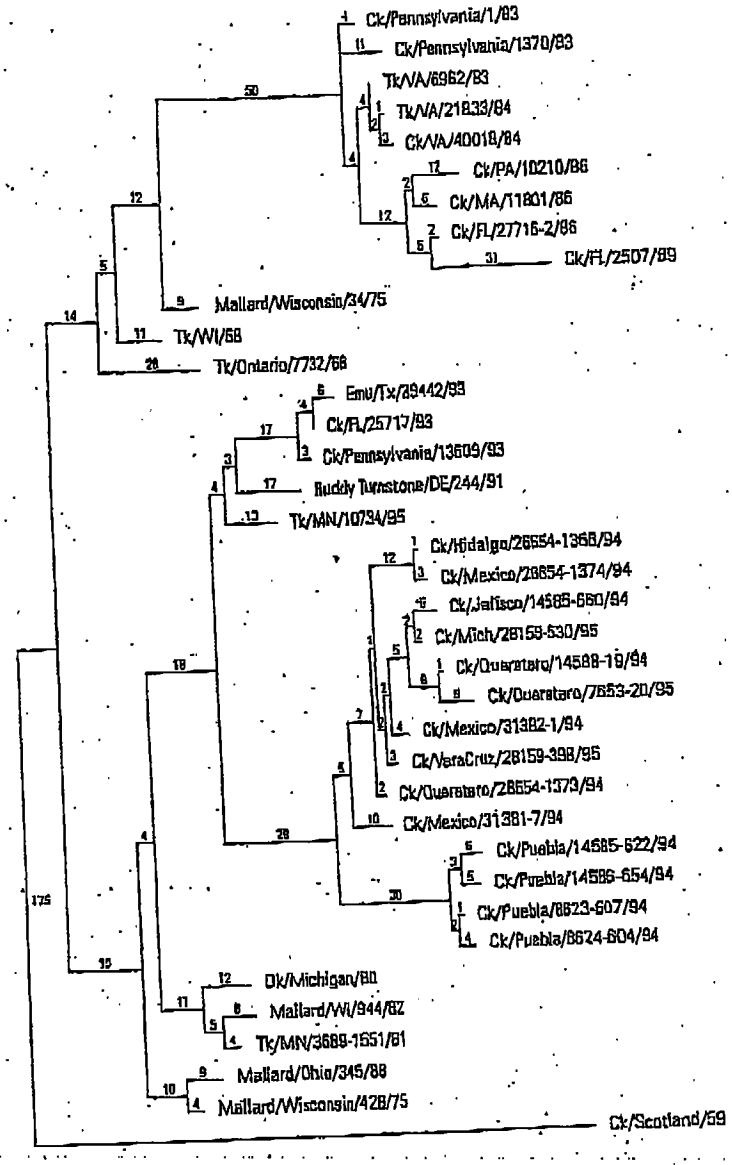


Fig. 2
Phylogenetic analysis of the nucleotide sequence of the haemagglutinin HA1 subunit of representative H5 isolates from North America
The tree was generated using the phylogenetic analysis using parsimony (PAUP) 3.1 computer program using a heuristic search. The tree is midpoint rooted, and Ck/Scotland/59 is included as a representative Eurasian H5 haemagglutinin sequence. Three clusters of H5N2 viruses are presented in the tree including the Pennsylvania/83-89 lineage, the live bird market associated/93 lineage viruses and the Mexican/93-95 lineage. All three groups, although all H5N2 viruses, are phylogenetically distinct and are the result of separate introductions of virus into the poultry population

Pasteurella multocida. Definitive diagnosis of an HPAI virus requires the following:

a) virus isolation and identification, serological identification, or demonstration of AI viral antigens or nucleic acids, and

b) determination of pathogenicity.

Virus isolates must be distinguished from all other haemagglutinating agents, including some avian adenoviruses, Newcastle disease virus and other avian paramyxoviruses by use of the AGID test with known positive monospecific influenza A antiserum. Dual isolations of influenza virus and Newcastle disease virus are not unusual.

Diagnosis can be complicated by the presence of other micro-organisms, such as mycoplasmas. Standard laboratory techniques using specific antisera to neutralise other viruses or antibiotics to control bacterial growth are helpful in such situations. Classification of AI viruses as HPAI viruses requires inoculation of susceptible chickens and production of ≥75% mortality, sequencing of the HA cleavage site to identify multiple basic amino acids, or production of cytopathic effect in tissue culture without exogenous trypsin (115).

Public health implications

Influenza viruses are adapted to a host species with transmission occurring most frequently and with ease between individuals of the same species, such as transmission from human to human, pig to pig and chicken to chicken (105). Interspecies transmission of influenza occurs, most easily and frequently between two closely related host species where the adaptation process to a new host can occur rapidly, e.g. transmission from chicken to turkey or chicken to quail (105). Interspecies transmission between mammals and birds has occurred rarely, examples are transmission from chicken to human or wild waterfowl to pig. The majority of such transfers have produced single or isolated cases of infection with elimination from the population before adaptation or reassortment with host adapted influenza viruses can occur in the new host population. One exception to the adaptation rule has been the ease and frequency of transfer of swine H1N1 viruses to turkey breeder hens (66), but these are sporadic occurrences and have involved only MPAI viruses. Other factors, such as geographic restriction, intermixing of species, age and density of birds, weather and temperature also affect the ability of the AI virus to move within and between host species and affect the overall incidence of infections (105).

Three pandemics of influenza have occurred in the human population during this century, in 1918 (H1N1), 1957 (H2N2) and 1968 (H3N2) (80). The immediate source of the influenza viruses is unknown, but molecular data suggests that some of the influenza genes originated from the AI genetic reservoir and recombined with existing human-adapted influenza viruses (80, 121). These AI genes reassorted with genes from existing human-adapted influenza viruses to create new influenza viruses that were adapted rapidly to the human host and exposed to an immunologically naïve population.

Four incidences have been documented of single cases or small clusters of cases demonstrating direct transmission of influenza viruses from birds to humans. The first was a fowl plague-like virus (H7N7) isolated in 1959 from a forty-six-year-old man with hepatitis, following his return from a two month trip overseas. The virus was HP for chickens, but was only moderately pathogenic for the man, who recovered (31). The second case was reported as conjunctivitis caused by an MPAI virus (H7N7), in a forty-three-year-old woman (13, 55). The woman tended ducks that shared a lake with feral waterfowl. The illness was mild and the woman recovered completely. The third incident involved infection and hospitalisation of eighteen human patients following infections by HPAI viruses (H5N1) in Hong Kong during 1997 (20, 38, 99, 129). The patients ranged in age from one- to sixty-years-old and six patients died during hospitalisation. Serological evidence of infection in asymptomatic poultry farmers was reported, but the infection rate was low. The live poultry markets and farms in Hong Kong were depopulated of 1.4 million chickens and other poultry at the end of 1997 (76). This action prevented further cases of AI in humans and probably averted a recombination event between the avian and human influenza strains or adaptation of the H5N1 AI virus to humans. In the case-controlled study (68), exposure to live poultry a week before illness was associated with the H5N1 influenza, but eating or preparing poultry products was not associated with H5N1 influenza. The fourth incident of direct transmission of AI viruses from birds to humans involved infection of seven individuals with MPAI (H9N2); two children in Hong Kong and five patients in the People's Republic of China were affected (25, 74). All patients recovered. In these four incidences of transmission of AI to humans, the influenza viruses that infected the humans had all eight genes of avian origin, but transmission was limited because of inefficient spread from human to human. Transmission was probably from aerosol exposure to high levels of virus in the respiratory secretions or faeces of the poultry and not from preparing or consuming poultry meat or products.

These occurrences emphasise the interspecies transmission potential of some AI viruses. However, the interchange or spread of influenza viruses between humans and other species, including birds, has been an exceedingly rare occurrence in nature (99). Some evidence suggests that AI viruses have different potential for infecting and causing disease in humans in such rare instances. During the seventeen HPAI outbreaks (Table I), human infections were identified in association with only the Hong Kong H5N1 outbreak in 1997. While the largest number of birds involved in a single outbreak of HPAI was in 1983-1984 in the USA (Table I), attempts to demonstrate primary replication and recovery of the H5N2 AI virus from workers in depopulation crews were unsuccessful (15). Using an experimental laboratory mouse model to predict the replication and virulence potential of HPAI viruses for humans, three AI viruses from Hong Kong (A/human/HK/156/97, A/chicken/HK/220/97 and A/chicken/HK/728/97) were shown to replicate to high titres in the lungs and to be HP for mice without prior passage or adaptation in mice (32). The strains A/chicken/Scotland/1959 (H5N1), A/chicken/Italy/97 (H5N2), and A/chicken/Queretaro/7653-20/95 (H5N2) had much lower levels of pulmonary replication in the mouse model and caused neither illness nor death in the mice.

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A/turkey/England/91 (H5N1) caused mild illness and death in one of eight inoculated mice. These mouse studies suggest that only certain AI viruses have the potential to cross from birds to mammals. Furthermore, no association has been found between the human cases of AI and the ability of the AI viruses to be HP or MP for chickens. Other workers have suggested a greater potential of influenza viruses to cross from pigs to humans (53), and pigs may be the intermediate mixing vessel of avian and mammal influenza viral genes (83).

The probability of an avian virus entering the human population, reassorting and establishing a new lineage of human influenza virus capable of causing a pandemic is extremely low, but this is consistent with the time span between emergence of the three human pandemic influenza viruses of the 20th Century (13). The main threat of AI viruses is not the direct spread of AI virus to the human host and in *toto* adaptation to the new human host, but the recombination of the AI virus with human-adapted influenza viruses that would lead to an antigenic shift and emergence of an influenza virus which could cause a pandemic in humans (13).

Prevention, control and eradication

Various strategies have been used for prevention, control and eradication of AI throughout the world. Strategies utilised cover the spectrum from no action on some MPAI viruses to the extreme of implementing stamping-out or depopulation programmes for eradication of HPAI (39, 106). Historically, individual countries have chosen control or eradication requirements to meet domestic expectations (57). However, the implementation of international trade agreements and standardisation of sanitary health requirements has challenged this approach. Measures taken to prevent, control or eradicate AI will vary depending on the pathogenicity of the AI viruses, types of birds infected, geographic distribution of infected birds, requirements of domestic and international markets and economic status of the nation (39, 102, 106). With regards to HPAI, prevention should be limited to strategies to preclude introduction and exposure of poultry to the virus. However, if HPAI does occur, eradication is the only viable option. In contrast, control implies reduction in incidence to a low or economically manageable level. A control programme may be an acceptable strategy for some strains of MPAI, but is not an acceptable option for an OIE List A disease such as HPAI or a highly virulent form of Newcastle disease. For MPAI or HPAI, an effective control or eradication programme has the following essential elements:

a) comprehensive, integrated national surveillance and diagnostic programmes

b) enhanced biosecurity practised at all levels of production and processing by all employees of companies, diagnostic laboratories and government agencies that have contact with poultry or equipment from poultry operations

c) education of poultry farmers and other workers about AI control and sharing of information on surveillance and control strategies at all levels in the production process

d) quarantine or controlled movement of AI-infected poultry

e) stamping-out or slaughter programme for all HPAI and some H5 and H7 MPAI outbreaks

f) vaccine use as one element of a comprehensive control programme and under specific situations with national government control.

Surveillance and diagnostics

A comprehensive, integrated surveillance and diagnostic programme is essential to determine the magnitude of AI virus infections in commercial and live-bird market domestic poultry, and migratory and non-migratory wild birds. Such information is essential to establish the national or regional prevalence of MPAI and HPAI within a country, which in turn affects the import and export potential of poultry, poultry products and other related avian commodities. Transparency of surveillance data between countries is essential in supporting international trade in poultry and poultry products. A national or regional veterinary-medical diagnostic infrastructure is essential to make rapid and accurate diagnosis of AI and to characterise the virus isolates as MP or HP. The rapid diagnosis of the index case of HPAI is crucial, as this will allow a rapid response to eliminate the disease before additional birds are infected and the disease becomes endemic. An example of such an infrastructure for surveillance and diagnostics exists in Australia, where in 1976, 1985, 1992, 1995 and 1997, limited outbreaks of HPAI occurred (Table I) (124). The presence of comprehensive veterinary diagnostic services in provincial and national government laboratories in addition to specially trained private and government veterinarians resulted in rapid diagnosis of HPAI and swift action by the national government to depopulate infected and exposed poultry resulting in the eradication of the disease early in the outbreak.

Biosecurity

Enhanced biosecurity is an important part of any AI prevention and control programme, and serves two purposes, as follows:

a) containment of the AI virus on infected farms (biocontainment)

b) prevention of introduction of the virus to naïve farms (bioexclusion).

The practice of biosecurity must be implemented at all levels within the poultry industry and allied groups, and by all employees involved. This includes personnel within the production companies, diagnostic laboratories and government agencies that have contact with poultry, poultry

products, poultry waste, equipment or other resources used on poultry farms. Proper disinfection and decontamination are essential for all equipment used on more than one farm, and common personnel should take the correct precautions to avoid carrying virus from faecal and respiratory secretions on boots, shoes and clothing. Ideally, workers should be restricted to one farming operation, and workers should not visit or work on other poultry farms and not own or tend poultry at their homes. The strength of a biosecurity programme does not lie in the written policy, but in the practice of biosecurity, from the simplest detail, by every individual concerned, and on all farms. When workers must be shared between farms, such as vaccination crews, in addition to depopulation and other crews, the highest level of biosecurity must be practised. Failure to do so presents a real danger as reported in eradication efforts against an outbreak of virulent Newcastle disease during the 1970s in California. The vaccination crews were epidemiologically linked to prolonging the outbreak by spreading the field virus between farms (116).

Education

Education and dissemination of information to poultry farmers and other workers, veterinarians, government regulators and the media, concerning AI status and required biosecurity practices are essential for the acceptance, implementation and successful outcome of a control programme. Failure to communicate critical information will encourage complacency and failure to comply or participate in the control efforts. Failure to provide information or the presentation of inaccurate information to the media will reduce public trust in the safety and wholesomeness of all poultry products for human consumption. Especially important is communication of the low potential for transmission of AI to people through consumption of poultry products.

Quarantine

Containment of the infection in individual farms or regions (zones), through controlled movement of poultry, equipment and personnel is essential in preventing spread of AI to non-infected locations. During outbreaks of HPAI, birds should be depopulated and disposed of within the quarantine zone. Disposal should be by incineration, composting, alkaline hydrolysis, fermentation or burial in accordance with environmental standards and laws. All equipment should be cleaned and disinfected before being removed from the infected farm. In some situations, controlled movement of poultry infected with MPAI may be necessary to minimise financial losses to farmers. Under such circumstances, the birds should be moved only after the infection has subsided and virus excretion titres are low, usually a minimum of three weeks after the first appearance of clinical signs of AI. Birds should be moved and processed at the end of the day. Transportation should be along routes that avoid poultry farms as much as possible.

Depopulation

In the case of outbreaks of HPAI, a stamping-out or slaughter programme is the best first line of defence for halting spread and eliminating the disease. The EU, USA, Australia and other countries, as well as the OIE, have policies that dictate depopulation as the principal control method for use in outbreaks of HPAI and other List A diseases of the OIE (5, 47, 73, 124). In addition, a depopulation programme may be necessary in outbreaks of low virulence H5 and H7 AI depending upon the impact on export of poultry products or the potential for MPAI viruses to mutate and become HPAI viruses (131). However, stamping-out programmes must be initiated early in an outbreak to be effective and economical, and these programmes tend to be expensive and subject to institutional inertia (106). The larger the geographic region involved and the greater the density of poultry, the greater the expense and difficulty in using a depopulation programme for eradication. In 1983-1985, the H5N2 HPAI outbreak cost the government of the USA US$63 million to eradicate, including the depopulation of 17 million birds (Table I) (59), while the eradication of H5N1 HPAI in Hong Kong during 1997 cost the government of Hong Kong HK$100 million including depopulation of 1.4 million birds (US$12 million) (L. Sims, personal communication, 12 September 1999).

Vaccines

Vaccination should be used only as one element of a comprehensive eradication programme for HPAI. Historically, AI vaccines have been used mostly in the USA as part of a control programme against sporadic outbreaks of MPAI in meat turkeys in the upper Midwest (39). Between 1979 and 1997, 22,385,000 doses of inactivated AI vaccines were used, but generally vaccines against H5 and H7 subtypes have been discouraged by the United States Department of Agriculture (40). A common use of vaccines has been to protect turkey breeders against sporadic outbreaks of MP swine H1N1 influenza viruses (79).

Although vaccines have been demonstrated to be protective against fowl plague in experiments, vaccination was not used in control and eradication programmes against HPAI until 1995, in Mexico and Pakistan (Table I) (36, 69). Since June and August 1995, HPAI has not been identified in Mexico or Pakistan, respectively. However, in Mexico, vaccines have continued to be used to control MPAI (H5N2) in broiler chickens, with 1.2 billion doses of inactivated AI vaccine and 0.3 billion doses of recombinant fowl pox vaccine with AI H5 gene insert being used between 1995 and 1999 (36). The government of the USA has provisions for usage of vaccines in future eradication efforts should HPAI appear in the USA (47). However, unrestricted use of AI vaccines will be counterproductive to elimination efforts unless the other five components listed earlier are integrated into the eradication programme (106). In general, vaccines should be used only if the number of HPAI cases is increasing and the disease is not

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contained within a single quarantine zone. Vaccine application should be limited to poultry within quarantine zones, barrier or 'ring' vaccination around the periphery of the quarantine zone, or vaccination to protect poultry pedigree stock within a region during an expanding HPAI outbreak. The difficulty with vaccine usage is deciding when to stop vaccination in order to conduct the final steps of the stamping-out programme.

Two vaccine technologies have been used in the control or eradication of HPAI, as follows:

a) inactivated whole virus vaccines

b) a recombinant fowl pox vaccine with an H5 AI HA gene insert.

Inactivated whole virus vaccines were used extensively in Mexico and Pakistan during the recent outbreaks involving H5 and H7 HPAI viruses, respectively (36, 69). The recombinant fowl pox vaccine has been used in Mexico in the control programme against MPAI (H5N2). Other technologies hold promise for future use, including deoxyribonucleic acid (81) and subunit HA protein vaccines (27).

The HA protein elicits the major protective immunity in poultry, but the NA also contributes to protection (33, 62). Vaccines provide protection in chickens and turkeys against illness and death following challenge by homologous HA subtypes of HPAI viruses; i.e. H5 vaccine protects against an H5 challenge virus, but not against an H7 challenge virus (22, 96, 97, 125, 127). This HA subtype protection is consistent between vaccine and field virus with at least 87% similarity in HA amino acid sequence (110). Haemagglutinin subtype-specific vaccines have been shown to lower infection rates and reduce quantity of challenge virus shed from cloaca and oropharynx of vaccinated chickens, but prevention of replication or 'sterilising immunity' has not been achieved (106, 109, 122, 127). Vaccines can provide protection against HPAI following a single dose for over twenty weeks (106, 109). Immunity induced by vaccines can provide protection even against challenge with high doses of HPAI virus. For recombinant fowl pox H5 AI viral gene insert vaccine, chickens three weeks post-vaccination were protected against illness and death when exposed to an HPAI virus from Mexico (A/chicken/Queretaro/14588-19/95 [H5N2]), even to challenge doses reaching $10^{7.5}$ mean chicken embryo lethal doses (ELD_{50}) per chicken (104).

Usage of inactivated AI vaccine can interfere with serological surveillance. Inactivated AI vaccines produce high antibody titres in poultry that are indistinguishable from field virus challenge as determined by the HI and agar gel precipitin (AGP) tests. To prevent interference with surveillance, unvaccinated sentinel birds should be placed within vaccinated flocks (39), or vaccines that do not elicit antibody responses to internal virus proteins should be utilised. Such vaccines include recombinant fowlpox with an AI HA gene insert or HA subunit protein vaccines that lack the nucleoprotein and matrix components and thus do not give an AGP response (27, 106).

Vaccine usage for AI control and eradication has definable limits. Experimental studies have shown good or excellent protection against MPAI and HPAI in SPF chickens, but in the complexity of the field situation, vaccine use and protection will not reach maximum potential. In the field, improper vaccination technique, infections by immunosuppressive viruses, incorrect storage and handling of vaccines and other factors make field vaccinated chickens and turkeys less well protected than those in laboratory studies. In control or eradication of AI, emphasis should be placed on vaccination as only one of the elements required in a comprehensive strategy. Otherwise, any gains achieved through vaccine protection from illness, death or reductions in shedding rates will be nullified. Control or eradication of AI can be achieved only if all aspects of the intervention strategy are operational. This includes strict biosecurity practices on infected and non-infected farms involving all personnel and equipment moved between farms, limiting human access to farms, providing adequate surveillance and diagnostics, imposing quarantine on infected flocks, and providing a low-risk method of elimination and disposal of infected birds.

As an alternative to vaccines, antiviral drugs have been proposed for treatment of avian influenza viral infections in poultry (120). However, in experimental studies simulating field conditions with HPAI, resistance to amantadine developed rapidly, calling into question the value of antiviral drugs for treatment of influenza in poultry (17). Furthermore, the cost of the drugs would be prohibitive, and in the USA, no antiviral drugs are approved by federal regulatory agencies for treatment of influenza in poultry. Poultry treated with antiviral therapy cannot be used for human consumption.

Measures to prevent importation of avian influenza

Based upon accepted OIE sanitary standards for List A diseases, the presence of HPAI typically prevents an affected country from exporting poultry and poultry products (73). Such movement restrictions are instituted by importing countries as legitimate measures to prevent the importation of HPAI. However, any restriction imposed by an importing country should be justified by a science-based risk analysis shared between the trading partners. For example, continued trade with an HPAI-free region of an exporting country could be accepted, despite the presence of HPAI in another region of that country, if adequate risk reduction movement controls are in place within the country.

Demonstration of freedom from HPAI can be accomplished through serological surveillance. Negative results in serological tests such as the AGID test or ELISA provide

reliable evidence of HPAI-free status. However, positive results in these serological tests can result from infections by either MPAI or HPAI viruses. To avoid trade restrictions, an exporting country must verify through virus isolation and *in vivo* and/or *in vitro* pathotyping tests that any positive serological results are from infections with MPAI viruses, especially if the viruses are H5 or H7 subtypes. The risk of importing MPAI in meat products is negligible because MPAI viruses replicate in the digestive and respiratory tracts, but not in meat tissue. In contrast, HPAI is a systemic disease and the virus can be present in most tissues, including meat.

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Influenza aviaire hautement pathogène

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Résumé

L'influenza aviaire hautement pathogène est une maladie extrêmement contagieuse de la volaille, à tropisme multiple et systémique, qui s'accompagne d'une mortalité élevée. Elle est due à certains sous-types H5 et H7 du virus influenza de type A, de la famille des *Orthomyxoviridae*. Toutefois, la plupart des souches virales de l'influenza aviaire sont modérément pathogènes et provoquent soit des infections infracliniques, soit des maladies respiratoires et/ou de la reproduction chez plusieurs espèces d'oiseaux domestiques et sauvages. L'influenza aviaire hautement pathogène fait partie de la Liste A de l'Office international des épizooties (OIE), alors que l'influenza aviaire modérément pathogène ne figure ni sur la Liste A ni sur la Liste B de l'OIE. Dix-huit épisodes d'influenza aviaire hautement pathogène ont été décrits depuis que l'origine de la peste aviaire de 1955 a été imputée au virus de l'influenza aviaire.

Les virus de l'influenza aviaire modérément pathogène sont présents chez les oiseaux migrateurs aquatiques, qui servent de réservoir ; ces virus atteignent occasionnellement des volailles domestiques, entraînent l'apparition de maladies peu sévères. Les virus de l'influenza aviaire hautement pathogène n'ont pas de réservoir reconnu chez les oiseaux sauvages, mais ils peuvent parfois être isolés chez ces derniers lors d'épidémies affectant les volailles domestiques. D'après la documentation existante, les virus de l'influenza aviaire hautement pathogène proviendraient des virus de l'influenza aviaire modérément pathogène suite à des mutations de la protéine de surface de l'hémagglutinine.

La stratégie recommandée en cas d'influenza aviaire hautement pathogène consiste à éviter toute exposition au virus et à éradiquer la maladie. Les programmes de prophylaxie qui tolèrent une faible incidence de l'infection ne constituent pas une méthode acceptable pour faire face à des cas d'influenza aviaire hautement pathogène, mais ils ont été utilisés lors de certaines épidémies d'influenza aviaire modérément pathogène. Les stratégies de lutte contre l'influenza aviaire hautement et modérément pathogène, reposent essentiellement sur le diagnostic, l'hygiène, l'éducation, la quarantaine et la réduction de la taille des élevages. La vaccination a été utilisée dans certains programmes de prophylaxie et d'éradication de l'influenza aviaire.

Mots-clés

Influenza aviaire hautement pathogène – Maladies aviaires – Orthomyxovirus – Peste aviaire.